Differentiation of human bone marrow-derived cells into buccal epithelial cells in vivo: a molecular analytical study

Simon D Tran, Stanley R Pillemer, Amalia Dutra, A John Barrett, Michael J Brownstein, Sharon Key, Evgenia Pak, Rose Anne Leakan, Albert Kingman, Kenneth M Yamada, Bruce J Baum, Eva Mezey

Summary

Background Adult bone marrow-derived (BMD) cells could be used to repair damaged organs and tissues, but the intrinsic plasticity of these cells has been questioned by results of in-vitro studies suggesting that such cells might fuse with other cells giving the appearance of differentiation. We aimed to determine whether fusion events are important in vivo.

Methods To test whether BMD cells can colonise an epithelial tissue and differentiate there without fusion, we did in-situ hybridisation with Y and X chromosome probes labelled with 35-sulphur or digoxigenin, or labelled fluorescently. We did immunohistochemistry with anticytokeratin 13 along with fluorescence in-situ hybridisation to identify Y-chromosome positive buccal epithelial cells in cheek scrapings obtained from five females who had received either a bone-marrow transplant or an allogeneic mobilised peripheral-blood progenitor-cell transplant (enriched in CD34+ cells) from male donors.

Findings When examined 4–6 years after male-to-female marrow-cell transplantation, all female recipients had Y-chromosome-positive buccal cells (0·8–12·7%). In more than 9700 cells studied, we detected only one XXXY-positive cell (0·01%) and one XXY cell (0·01%), both of which could have arisen when an XY cell fused with an XX cell.

Interpretation Male BMD cells migrate into the cheek and differentiate into epithelial cells, an occurrence that does not depend on fusion of BMD cells to recipient cells. This finding might be an example of transdifferentiation of haemopoietic or stromal progenitor cells. Plasticity of BMD cells could be useful in regenerative medicine.

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National Institute of Dental and Craniofacial Research, Gene Therapy and Therapeutics Branch (S D Tran DMD, S R Pillemer MD, R A Leakan RN, B J Baum DMD); **Craniofacial Developmental Biology and Regeneration Branch** (K M Yamada MD); **Biostatistics Core** (A Kingman PhD); **National Human Genome Research Institute, Cytogenetic Core** (A Dutra PhD, E Pak MD); **National Heart Lung and Blood Institute, Hematology Branch** (A J Barrett MD); **National Institute of Mental Health, Laboratory of Genetics** (M J Brownstein MD); **National Institute of Neurological Disorders and Stroke, Basic Neuroscience Programme** (S Key BS, E Mezey MD); **National Institutes of Health, Bethesda, MD, USA**

Correspondence to: Dr Simon Tran, National Institutes of Health/NIDCR, 10 Center Drive, Room 1N113, MSC 1190, Bethesda MD 20892-1190, USA
(e-mail: stran@dir.nidcr.nih.gov)

Introduction

Evidence from studies in recipients of bone-marrow transplants shows that bone marrow-derived (BMD) cells can differentiate into cells other than blood. This occurrence is of importance because such cells could be used to regenerate organs after disease or trauma.[1–5] However, the plasticity of BMD cells has been questioned by results of studies suggesting that stem cells might fuse with other cells and give the appearance of differentiation.[6,7] Furthermore, caution is needed in interpretation of data purporting to show that non-haemopoietic cells are derived from donor BMD cells.[8–10] Tissues, especially when affected by graft-versus-host disease (GVHD), might contain infiltrating donor haemopoietic or lymphoid cells. In histological sections, non-haemopoietic tissue cells might be confounded with haemopoietic cells because of cell overlap (attributable to the thickness of the sections) or loss of haemopoietic lineage-specific markers.

We aimed to establish whether BMD cells differentiate into cells of another tissue lineage, and to assess whether this event is attributable to fusion. To avoid contamination of our samples by haemopoietic inflammatory cells, we did our studies on healthy transplant recipients without oral GVHD, several years after bone-marrow transplantation.

Methods

Patients

Bone-marrow stem-cell transplant recipients residing within the USA, who each had received an allogeneic transplant from a male sibling donor through protocols from the National Heart, Lung, and Blood Institute, were invited to participate in the study. All those who agreed to participate were well, in haematological remission, with full donor lymphohaemopoietic engraftment and no active oral GVHD. The transplants these patients had received consisted of at least 3×10^6 CD34+ cells/kg and a total of 1×10^7–3×10^8 CD3+ lymphocytes/kg given within 3 months of the stem cells. After the transplant, all patients received only irradiated blood products. We also recruited healthy volunteers as controls. This study was approved by the Institutional Review Board of the National Institute of Dental and Craniofacial Research (NIDCR), National Institutes of Health (NIH).

Procedures

We obtained cheek scrapings from all women to look for Y-chromosome-positive buccal epithelial cells. Study of buccal cells has the advantage of providing a non-invasively obtained, pure single-cell population of mature nucleated cells with a characteristic morphology and phenotype (anticytokeratin 13+). Cheek scrapings spread on glass slides were independently examined by two pathologists. We also gathered blood samples from these women.

	Recipient				
	1	2	3	4	5
Characteristic					
Age at transplant (years)	31	36	37	56	40
Reason for transplant	CML	MDS	AML	CML	CML
Type of transplant	BM	PBPC	PBPC	PBPC	BM
Chemotherapy/radiotherapy	Yes/Yes	Yes/Yes	Yes/Yes	Yes/No	Yes/Yes
Time from transplant to buccal samples (years)	5·2	4·5	4	4·2	6
History of pregnancy	Never	Never	1 daughter, 2 miscarriages	2 daughters, 1 son	2 daughters, 1 son, 1 miscarriage
History of blood transfusion	Never	Yes	Yes	Never	Never

AML=acute myelogenous leukaemia. BM=bone marrow transplant; CML=chronic myelogenous leukaemia; MDS=myelodysplastic syndrome; PBPC=peripheral-blood progenitor-cell allogeneic transplant (granulocyte-colony stimulating factor mobilised).

Table 1: **Characteristics of female transplant-recipients**

In situ hybridisation and immunohistochemistry

We did in-situ hybridisation as described,[3] with a digoxigenin-labelled riboprobe complementary to nucleotides 729–2053 of the Y-chromosomal marker DYZ1 (accession number X06228).[11] We prepared the template from human genomic DNA and produced a 1·3 kb riboprobe by T7 polymerase and either 35-sulphur-labelled or digoxigenin-labelled UTP. After hybridisation, the digoxigenin-labelled probe was visualised with a peroxidase-conjugated antidigoxigenin antibody (Roche, Indianapolis, IN, USA), followed by tyramide signal amplification with the FITC-tyramide plus reagent (TSA System, Perkin-Elmer, Boston, MA, USA). For autoradiographic detection of the Y chromosome, we used the method described elsewhere.[12] For double-labelling of cytokeratin and Y chromosome, buccal cells were fixed in a

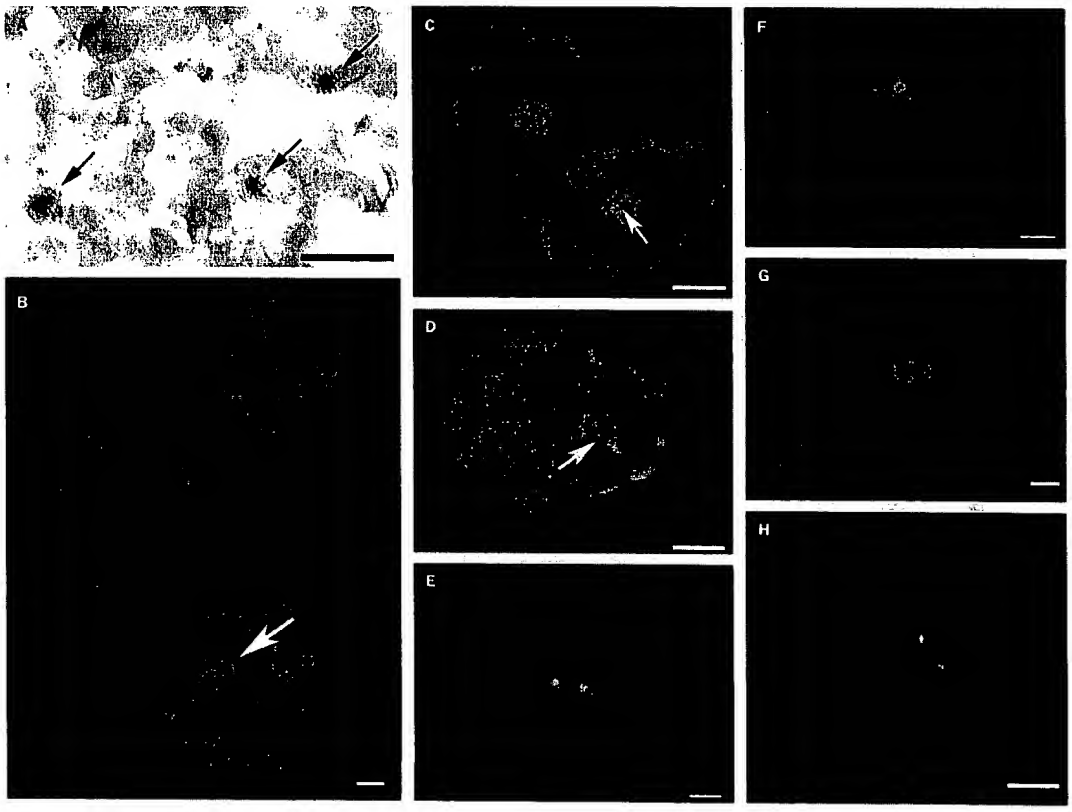

Figure 1: **In-situ hybridisation and immunohistochemistry in human buccal cells**

(A) Y-chromosome autoradiography on a smear of buccal cells from recipient 3 is shown in brightfield. The arrows point to the autoradiographic grains (black dots) above the nuclei of Y-chromosome containing cells. (B, C, D) Buccal cells from recipients 4, 2, and 3, respectively. The cells were first immunostained for cytokeratin 13 (green), then hybridised in situ with a human Y-chromosome riboprobe (red fluorescent dots shown by the arrows). The nuclei of the cells are stained blue with DAPI. (E, F, G, H) FISH with X-chromosome and Y-chromosome probes. Individual buccal cells are shown from a healthy female control (E), a healthy male control (F), recipient 3 (G), and recipient 1 (H). The green fluorescent dot in the nucleus represents the X probe and the red dot the Y probe hybridised to the appropriate regions of the sex chromosomes. The nuclei of the cells are stained blue with DAPI. The cytoplasm of cells in panels E–H are autofluorescent. The scale bar represents 10 μm in all panels, except 100 μm for panel A. An RGB version of this figure is available at http://image.thelancet.com/extras/02art10351webfigure.pdf.

	Recipient				
	1	2	3	4	5
S^{35}-radiolabelled Y probe					
Number of nuclei examined	3907	747	2658	966	1285
Number of Y-positive nuclei (%)	194 (5·0%)	13 (1·7%)	293 (11·0%)	13 (1·4%)	125 (9·7%)
FISH with X and Y probes					
Number of nuclei examined	1824	1779	1315	2853	1977
Number of Y-positive nuclei (%)	206 (11·3%)	42 (2·4%)	167 (12·7%)	23 (0·8%)	136 (6·9%)

Table 2: **Detection of Y-chromosome-positive cells in cheek scrapings from female transplant-recipients**

picric acid-aldehyde fixative for 15 min at room temperature, and we did immunocytochemistry first with a cytokeratin 13 antibody at a 1:1000 dilution (132M, BioGenex, San Ramon, CA, USA) followed by tyramide signal amplification (Perkin-Elmer). After immunostaining, we processed the slides for fluorescence in-situ hybridisation (FISH) and assessed them with a Leitz or Zeiss fluorescence microscope.[12]

FISH with X and Y chromosome probes

We placed buccal cells in Carnoy's fixative for 15 min at room temperature. A mixture of two centromeric probes—CEP X (alpha satellite DNA) labelled with spectrum green and CEP Y (satellite III DNA) labelled with spectrum orange (Vysis, Downers Grove, IL, USA)—was denatured at 74°C for 5 min and hybridised overnight at 37°C in a humidified chamber. We did post-hybridisation washes at 45°C in 50% formamide and 2×SSC for 15 min and 1×SSC for 15 min. We counterstained slides with 250 mg/L DAPI (4',6-diamidino-2-phenylindole dihydrochloride) in mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA).

Genotyping

DNA was extracted from the cheek cells and donor blood samples with PureGene reagents (Gentra, Minneapolis, MN, USA). We obtained markers from Research Genetics, amplified with TaqGold (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol, and analysed them on an ABI 3100 capillary sequencer. Allele sizing was done with Genotyper software (version 2.5, Applied Biosystems).

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Five females, 4–6 years after receiving a transplant from an HLA-identical brother for leukaemia, agreed to participate (table 1). Recipients 1, 2, 3, and 5 underwent a myeloablative conditioning regimen consisting of 4 days of total body irradiation (13·6 Gy) and 2 days of cyclophosphamide 60 mg/kg. Recipient 4 received the same dose of cyclophosphamide plus fludarabine 125 mg/m² over 5 days. GVHD developed in four: acute grade 1 in recipient 1; chronic liver GVHD in recipient 3; extensive chronic GVHD in recipient 4; and acute grade 4 in recipient 5. Only recipient 3 had active GVHD at the time of testing.

Cheek scrapings from these five women consisted of discrete epithelial cells without contamination by mononuclear cells or macrophages. The S^{35}-radiolabelled Y-chromosome probe hybridised in situ with 97·7% of the 5354 buccal cells obtained from four healthy male volunteers and none of the buccal cells obtained from ten female volunteers (500–1000 buccal cells were counted per female volunteer). Therefore, the sensitivity of the S^{35}-radiolabelled Y-chromosomal probe was 97·7% (95% CI 96·9–98·5) and the specificity was 100% (99·8–100).

In the five transplant-recipients, frequency of Y-positive nuclei in buccal epithelial cells ranged between 1·4% and 11% (mean 5·76; 95% CI 0·24–11·28; figure 1 A, and table 2). Presence of male cells was confirmed in recipient 5 by DNA genotyping of the donor's blood, the patient's buccal epithelial cells, and her son's blood cells, with Y-chromosomal markers 289, 388, 390, and 391; none of these markers gave products when female control DNA was used as template (figure 2). On the other hand, patient 5's donor's blood and her own cheek samples always had the same genotypes. The alleles for the above markers were 148, 126, 211, and 287, respectively. However, patient 5's son had alleles 148, 126, 215, and 283. This finding shows that the patient's Y-chromosome-positive cheek cells could not have arisen by microchimerism,[13] and the fact that the genotypes of the patient's buccal cells and donor's blood cells are identical strongly suggests that the former arose from the graft. We detected no Y-positive amplicons in untransplanted female control samples, and no Y-positive buccal cells in ten healthy control women who have sons. Combined staining for the Y-chromosome by FISH and by histochemistry for anticytokeratin 13 confirmed the presence of male buccal epithelial cells (figure 1 B, C, and D). Cryopreserved bone-marrow samples, available from the donors of recipients 1 and 5, were negative for cytokeratin 13, indicating no epithelial cells were present in the bone-marrow transplant specimen.

We next used X and Y chromosomal FISH to search for cell fusion. As controls we analysed 3693 female and 1855 male buccal cells obtained from two female and two male healthy volunteers (figure 1 E and F). The sensitivity of detection of the Y-chromosome probe was 98·6% (95% CI 96·8–100) and the specificity was 100% (99·9–100). The X-chromosome probe hybridised to two X chromosomes in 99·0% of 3693 female control buccal cells (sensitivity 99·0%; 95% CI 97·8–100). We analysed between 1300 and 2800 cells per female transplant-recipient. The overall frequency of Y-positive nuclei in cheek scrapings of female transplant-recipients varied from 0·8 to 12·7% (mean 6·81; 95% CI 0·28–13·34; figure 1 G and H, and table 2). Of 9748 cells examined from the five transplant-recipients, one cell (0·01%) was XXXY and one was XXY (0·01%).

Discussion

These results indicate that cells derived from marrow or granulocyte-colony stimulating factor mobilised blood cells migrate into the cheek and differentiate into epithelial cells. These results might therefore represent transdifferentiation of haemopoietic or stromal stem cells, or transplantation of a hypothetical epithelial progenitor cell.

Four criteria have been proposed to show plasticity of adult stem cells.[14,15] The first criterion states that cells grafted should be clonal. Our study did not fulfil this requirement because small populations of blood-derived or

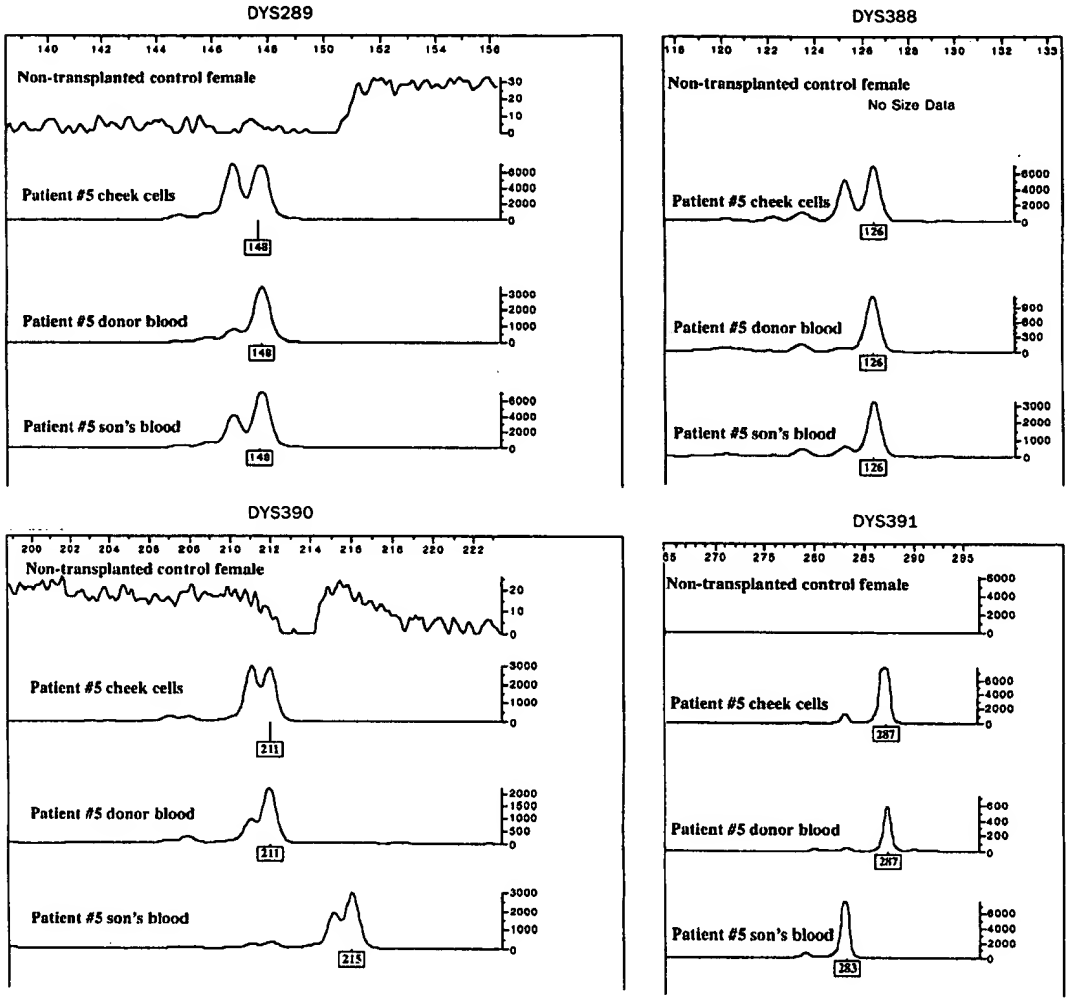

Figure 2: **DNA analysis**

Blood from a non-transplanted female control, buccal cells from recipient 5, her donor's blood, and her son's blood, were analysed with four sets of Y-chromosome markers. X axis shows product length in bases, and Y axis shows signal strength.

marrow-derived cells other than the CD34+ selected haemopoietic progenitors could have been transplanted. Therefore, the Y-chromosome positive buccal epithelial cells seen in our female transplant recipients could have originated from male donor haemopoietic stem cells, mesenchymal stem cells, or epithelial stem cells. Results of many reports suggest that both haemopoietic stem cells and mesenchymal stem cells can differentiate into epithelial cells.[5,16,17] However, results of some reports argue against the possibility that haemopoietic stem cells can differentiate into non-haemopoietic tissues.[18] Epithelial stem cells have not been described in the bone marrow, nor was cytokeratin 13 detected in the bone marrow of two male donors tested.

The second criterion for plasticity of adult stem cells requires that transplanted cells be prospectively isolated and transplanted without in-vitro culturing. Our study meets this criterion, since the sorted CD34+ fractions were transplanted without any in-vitro culturing.

The third criterion, which requires that the phenotype of the transdifferentiated cells be shown anatomically, molecularly, and functionally, is met: Y-chromosome positive buccal cells were morphologically distinguishable as buccal epithelial cells, and they expressed cytokeratin 13, a recognised epithelial marker located in the superficial buccal-cell layer of the cheek. Although the function of the differentiated cells could not be verified, the buccal mucosa of these individuals seemed unremarkable and intact.

The fourth criterion is to report the frequency of the transdifferentiation event. We noted that 0·8–12·7% of the nuclei studied were Y-chromosome positive. This high frequency of engraftment might be related to the rapid turnover rate (every 7–10 days) of the oral mucosa. Buccal epithelial cells obtained from cheek scrapings were dispersed on a glass slide. Thus, we could not establish whether the high frequency of transdifferentiation seen was attributable to clonal expansion of epithelial stem cells or

frequent transdifferentiation of individual graft-derived cells. To answer this question, one would need to obtain incisional biopsy specimens of the oral mucosa. Overall, this study meets three of the four proposed criteria for adult stem-cell plasticity.

A second important finding is that fusion between donor and recipient cells was a rare occurrence in vivo, just as it is in vitro.[6,7] With one or two exceptions, the greater than 9700 differentiated buccal cells seen in this study showed no evidence of fusion. Thus, differentiation was not attributable to host and donor cell fusion. Although the clinical implications of adult bone-marrow cell plasticity are still unclear, focusing on non-fusion mechanisms by which cell fate is ascertained in man is likely to benefit the areas of cell therapy and regenerative medicine.

Contributors

S D Tran, S R Pillemer, B J Baum, and E Mezey wrote the protocol for this clinical study. They were assisted in haematological consultation and management by A J Barrett, in cell biology consultation by K M Yamada, and in statistical consultation by A Kingman. A Dutra and E Pak provided the FISH X/Y results. E Mezey and S Key provided the in-situ hybridisation and immunohistochemistry results. M J Brownstein provided the DNA genotyping results. R A Leakan managed and coordinated the patients enrolled in this study. All authors were involved in the preparation of this report and agreed to its final version.

Conflict of interest statement

None declared.

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References

1 Horwitz EM, Gordon PL, Koo WK, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc Natl Acad Sci USA* 2002; 99: 8932–37.

2 Horwitz EM, Prockop DJ, Fitzpatrick LA, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999; 5: 309–13.

3 Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* 2000; 290: 1779–82.

4 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; 410: 701–05.

5 Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002; 418: 41–49.

6 Terada N, Hamazaki T, Oka M, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 2002; 416: 542–45.

7 Ying QL, Nichols J, Evans EP, Smith AG. Changing potency by spontaneous fusion. *Nature* 2002; 416: 545–48.

8 Abkowitz JL. Can human hematopoietic stem cells become skin, gut, or liver cells? *N Engl J Med* 2002; 346: 770–72.

9 Brouard M, Barrandon Y. Male cells in female recipients of hematopoietic-cell transplants. *N Engl J Med* 2002; 347: 218–20.

10 Fogt F, Beyser KH, Poremba C, et al. Recipient-derived hepatocytes in liver transplants: a rare event in sex-mismatched transplants. *Hepatology* 2002; 36: 173–76.

11 Nakahori Y, Mitani K, Yamada M, Nakagome Y. A human Y-chromosome specific repeated DNA family (DYZ1) consists of a tandem array of pentanucleotides. *Nucl Acids Res* 1986; 14: 7569–80.

12 Laboratory of Cellular and Molecular Recognition, National Institute of Mental Health. Section on Neural Gene Expression. http://intramural.nimh.nih.gov/lcmr/snge/ (accessed Jan 24, 2003).

13 Nelson JL. Microchimerism: incidental byproduct of pregnancy or active participant in human health? *Trends Mol Med* 2002; 8: 109–13.

14 Anderson DJ, Gage FH, Weissman IL. Can stem cells cross lineage boundaries? *Nat Med* 2001; 7: 393–95.

15 Wells WA. Is transdifferentiation in trouble? *J Cell Biol* 2002; 157: 15–18.

16 Clarke D, Frisen J. Differentiation potential of adult stem cells. *Curr Opin Genet Dev* 2001; 11: 575–80.

17 Verfaillie CM. Adult stem cells: assessing the case for pluripotency. *Trends Cell Biol* 2002; 12: 502–08.

18 Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* 2002; 297: 2256–59.